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ORIGINAL ARTICLE

Phenotypic and Genotypic Methods for Detection of Metallo-Beta-Lactamase (MβL) Producing *Pseudomonas Aeruginosa*

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Aeruginosa,
MβL,
IMP-EDTA CDT,
PCR

ABSTRACT

Background: *The occurrence of a MβL-positive isolate in a hospital setting represents a therapeutic problem, as well as a serious concern for infection control management. The accurate identification and reporting of MβL-producing P. aeruginosa will aid infection control practitioners in preventing the spread of these multidrug-resistant isolates.* ***Objectives:*** *This study aimed to detect and characterize MβL producing P. aeruginosa in Benha and to evaluate IMP- EDTA CDT as phenotypic screening method for MβL detection.* ***Methodology:*** *This study was conducted on 100 P.aeruginosa strains isolated from 220 different clinical specimens collected from patients admitted to Benha University Hospital and Benha Teaching Hospital. The isolated P. aeruginosa strains were subjected to antibiotic susceptibility testing by disc diffusion test and MβL detection both phenotypically by IMP- EDTA CDT and genotypically by multiplex PCR.* ***Results:*** *Out of 100 P.aeruginosa isolates, 25 strains (25%) were imipenem resistant and 15 strains of them (60%) were carrying genes responsible for MβL production (15% of the total number of P.aeruginosa). Thirteen strains (13%) were carrying VIM gene while two strains (2%) were carrying both VIM and SPM genes together. IMP, GIM-1, SIM-1 genes were not detected. None of the imipenem sensitive strains were carrying genes of MβL production. Nearly all MβL producers were resistant to most antibiotics used while all strains were sensitive to colistin and polymyxinB. There is very good strength of agreement between IMP-EDTA CDT and PCR. The sensitivity & specificity of the IMP-EDTA CDT in relation to PCR was 93.3% and 100% respectively.* ***Conclusion:*** *MβL producers is a serious problem as they are highly resistant to most antibiotics used making treatment options very limited, VIM gene is the most prevelant one in comparison with other genes of MβL production and IMP-EDTA CDT is a good and sensitive test in detecting MβL production.*

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is a versatile nosocomial opportunist, often multiresistant to antibiotics. *P. aeruginosa*, a non-fermenting Gram-negative rod of great clinical and epidemiological relevance in hospital-acquired infections, is more frequently found in intensive care units and is associated with high morbidity and mortality [1].

The increasing rates of antibiotic resistance among *P. aeruginosa* are of serious concern. *P.aeruginosa* have significant intrinsic resistance to antibiotics. Antipseudomonal beta-lactams (β-lactams) such as ticarcillin, piperacillin, ceftazidime, cefepime, aztreonam, and the carbapenems have an important therapeutic value [2].

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Resistance to β-lactams may be mediated by upregulated chromosomal (AmpC) beta-lactamases (β-lactamases), efflux or porin loss or by acquired β-lactamases, included extended spectrum beta lactamases (ESβLs) and carbapenemases [3].

Carbapenem antibiotics are considered the last resort for the treatment of serious infections. These agents have the broadest antibacterial spectrum compared to other β-lactam classes such as penicillins and cephalosporins. Additionally, they are generally resistant to the typical bacterial β-lactamase enzymes which are one of the principal resistance mechanisms of bacteria. They are active against both Gram positive and Gram negative bacteria, with the exception of intracellular bacteria, such as Chlamydiae [4].

Two types of carbapenemases are recognized; serine β-lactamases (having serine at their active site) and the metallo-β-lactamases (MβLs) which contain a metal ion that works as a cofactor for the enzyme's activity [5].

The (MβLs) have emerged as one of the most feared resistance mechanisms because of their ability to hydrolyze virtually all β-lactam agents, including the carbapenems, and because their genes are carried on highly mobile elements. Moreover, MβLs are not susceptible to therapeutic β-lactamase inhibitors [6].

Over the last decade MβL producing isolates have emerged particularly in *P. aeruginosa*. These isolates have been responsible for serious infections such as septicemia and pneumonia and have been associated with failure of therapy with carbapenems. In last years, MβL genes have spread from *P. aeruginosa* to Enterobacteriaceae, and a clinical scenario appears to be developing that could simulate the global spread of extended-spectrum β-lactamases. It is well known that poor outcome occurs when patients with serious infections due to MβL producing organisms are treated with antibiotics to which the organism is completely resistant [7].

Based on amino acid sequence homology, multiple types of MβL genes had been recognized; IMP (imipenemase), VIM (Verona integron-encoded MβL), SPM (Sao Paulo MβL), GIM (German imipenemase), SIM (Seoul imipenemase), NDM-1 (New Delhi MβL), KHM-1 and DIM-1 (Dutch imipenemase) [8].

The problem is aggravated by the fact that most of the MβL-encoding genes reside on integrons and plasmids which in turn allows for the widespread dissemination of these genetic elements[9]. Poor therapeutic outcomes and increased mortality rates have been associated with infections of such organisms. Therefore, early detection of MβL-producing organisms is of crucial importance to permit rapid initiation of strict infection control procedures, allow timely institution of effective therapy and prevent nosocomial spread [10].

METHODOLOGY

Patients and samples:

The study was conducted on 100 strains of *P.aeruginosa* isolated from 220 random clinical samples including: bronchoalveolar lavage (BAL), sputum, wound swab, infected burn wound swab, urine, stool, blood, ear swab and eye swab. The clinical samples were collected from 220 hospitalized inpatients admitted to Benha University hospital and Benha Teaching Hospital, they were 130 males and 90 females and their ages ranged from days to 80 years.

This study was approved by Benha University ethical committee and consent was obtained from all patients under study.

Isolation and identification of *P. aeruginosa*:

Clinical samples were cultured directly on Pseudomonas Cetrimide Agar**.** Blood samples were inoculated into blood culture bottles (Egyptian Diagnostic Media) then incubated at 37 °C for 7–14 days. Subcultures were done every 48 h on Pseudomonas Cetrimide Agar.The plates were incubated at 37°C for 1-2 days and then the growing organisms were identified as *P. aeruginosa* by the standard laboratory technique including: Gram staining, colony morphology, motility, pigment production, oxidase reaction, growth at 42 °C, Gelatin liquefaction test and Sugar utilization tests.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was performed on Mueller Hinton agar plates by Kirby–Bauer disc diffusion method and interpreted according to Clinical Laboratory Standards Institute recommendations (CLSI)[11].

The discs used were: amikacin (30 ug), gentamycin (10 ug) , tobramycin (10 ug), piperacillin+tazobactam (100/10 ug), meropenem (10 ug), imepinem (10 ug), ceftazidime (30 ug), cefoperazone (10 ug), cefotriaxone (30 ug),cefotaxime (30 ug), cefepime (30 ug), aztreonam (30 ug), amoxycillin/clavulanic acid 2:1(30 ug), ciprofloxacin (5 ug), polymixin B (10 ug) and colistin (10 ug).

Phenotypic detection of MβL activity:

All *P. Aeruginosa* isolates resistant to imipenem were investigated for MβL production by imipenem + EDTA combined disc test (IMP-EDTA CDT) as described previously by *Yong et al* [12]. Briefly, an overnight culture of the test organism was diluted with peptone water to 10^5 CFU/mL and inoculated on plates with Mueller Hinton agar plate using cotton swab**.** Two imipenem discs (10 μg) were placed at a distance of 4-5 cm from each other on the plate, and appropriate amounts of 10 μl of 0.5 M EDTA solution were added to one of them. The inhibition zones of the imipenem and imipenem-EDTA discs were compared after 16 to 18 hours of incubation aerobically at 35°C. Isolates with enhancement of zone size of more than or equal to 7 mm between imipenem+EDTA disc compared to Imipenem disc alone were considered as MβL positive (Figure 1).

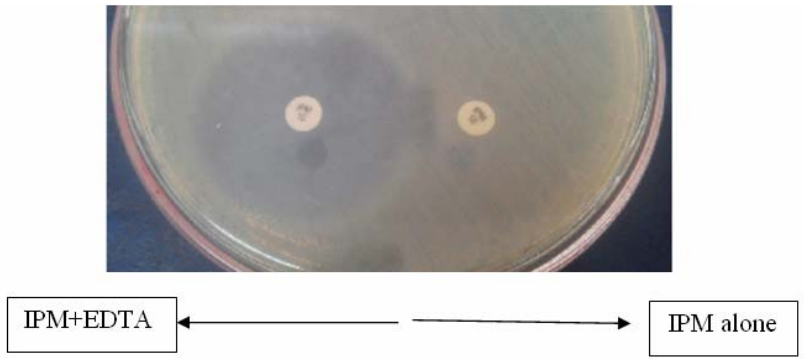

Fig. 1: A plate of Muller Hinton agar showing imipenem EDTA combined disc test indicating a MβL producing *P. aeruginosa* strain.

Multiplex PCR for detection of MβL genes:

Multiplex PCR testing of all isolates for MβL genes was done according to *Ellington et al* [13] to detect each of the five families of acquired MβL genes in a single reaction. Five primer pairs (Biosearch technologies, USA), specific for each family of acquired MβLs listed in *(Table 1)* were used.

DNA extraction:

Total DNAs of the different bacterial isolates were extracted by the DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721) according to manufacturer instructions. The extracted DNA was then stored at -20°C until further processing.

DNA amplification:

Amplification was done using Maxima Hot Start PCR Master Mix #K1051 (Thermo Scientific, EU Lithuania). The PCR mix contained 25ul of PCR master Mix, 1ul of each forward primer, 1ul of each reverse primer, 5ul of the template DNA and the amount completed with nuclease free water to reach a final volume of 50ul. Two PCR reactions were made for each sample. Reaction 1 (M1) was containing three primers (SIM-1, SPM-1, and GIM-1) while reaction 2 (M2) was containing two primers (IMP and VIM).*G storm* thermal cycler Uk was used for amplification according to the following program: initial denaturation at 95 ºC for 4mins, 45 cycles of denaturation at 95 ºC for 30 s, annealing at 46 ºC for 30 s and extension at 72 ºC for 45 s, followed by final extension at 72 ºC for 10 mins.

DNA detection by agarose gel electrophoresis:

Fifteen µl of each amplified DNA & 100bp ladder (molecular weight marker) were separated on 2% agarose gel containing 0.3 mg/ml of ethidium bromide. The bands were visualized using UV transilluminator (312 nm), photographed & analyzed [14].

Table 1: Sequence of primers used in amplification of MβL genes [13].

Primer	*Primer sequence 5'---------- 3'*	*Product length*
IMP family		
Imp-F	5'-GGA ATA GAG TGG CTT AAY TCT C-3'	
Imp-R	5'-CCA AAC YAC TAS GTT ATC T-3'	188 bp
VIM family		
Vim-F	5'-GAT GGT GTT TGG TCG CAT A-3'	390 bp
Vim-R	5'-CGA ATG CGC AGC ACC AG-3'	
GIM-1		
Gim-F	5'-TCG ACA CAC CTT GGT CTG AA-3'	
Gim-R	5'-AAC TTC CAA CTT TGC CAT GC-3'	477 bp
SPM-1		
Spm-F	5'-AAA ATC TGG GTA CGC AAA CG-3'	
Spm-R	5'-ACA TTA TCC GCT GGA ACA GG-3'	271 bp
SIM-1		
Sim-F	5'-TAC AAG GGA TTC GGC ATC G-3'	570 bp
Sim-R	5'-TAA TGG CCT GTT CCC ATG TG-3'	

Statistical analysis:

Data were recorded and analyzed using the computer program SPSS (Statistical package for social science) version 16 to obtain descriptive data in the form of number and percent. Kappa test measure the level of agreement between IMP EDTA CDT and PCR.

RESULTS

The results of this study show that the highest isolation rate of *P. aeruginosa* was from BAL samples (48%) while no *P.aeruginosa* strains were isolated from sputum, blood or stool samples (Table 2).

Out of 100 strains of *P. aeruginosa*, 25 isolates (25%) were resistant to imipenem. Nearly 100% of *P. aeruginosa* strains were resistant to cefotaxim, cefotriaxone, cefoperazone, ceftazidime, cefepime and amoxicillin- clavulanic acid while 100% of them were fully sensitive to colistin and polymyxin B (Table 3).

PCR was done for all *P. aeruginosa* isolates and the results revealed that out of 25 imepinem resistant *P. aeruginosa* isolates, 15(60%) strains were carrying genes for MβL production [13 (52%) strains were carrying *VIM* gene and 2 strains (8%) were carrying *SPM* and *VIM* genes together]. None of Imipenem sensitive strains were carrying MβL genes (Table 4) (Figure 2).

IMP-EDTA CDT was done for all 25 Imipenem resistant isolates and the results revealed that 14 out of 25 (56%) *P. aeruginosa* isolates were positive (MβL producer) (Table 5).

When comparing the results of IMP-EDTA CDT with the results of PCR, we found that the strength of agreement between them is considered to be very good*(Kappa value=0.92)(Table5)* and the sensitivity & specificity of the IMP EDTA CDT in relation to PCR was 93.3% and 100% respectively.

Nearly all MβL producers were resistant to most antibiotics used while 100% of them were fully sensitive to colistin and polymyxin B (Table 6).

Table 2: Number and percentages of *P. aeruginosa* strains isolated from different clinical samples.

Samples	*No. and percentages of different samples*	*No. and percentages of* **P.aeruginosa** *isolated from different samples*	*No. of* **P. aeruginosa** *isolated in relation to the total No. of strains*
BAL	80/220 (36.3%)	48/80 (60%)	48/100 (48%)
Sputum	13/220 (5.9%)	-	0
Blood	11/220 (5%)	-	0
Urine	23/220 (10.4%)	11/32(47.8%)	11/100 (11%)
Wound swab	33/220 (15%)	16/33(48.5%)	16/100 (16%)
Burn wound swab	31/220 (14%)	16/31(51.6%)	16/100 (16%)
Stool	2/220 (0.9%)	-	0
Eye swab	12/220 (5.4%)	5/12(41.6%)	5/100 (5%)
Ear swab	15/220 (6.8%)	4/15(26.7%)	4/100 (4%)
Total	220		100

Table 3: Antibiotic sensitivity pattern of all isolated strains of *P. aeruginosa*.

Antibiotic	*All Pseudomonas strains (100 strain)*		
	Resistant	**Intermediate**	**Sensitive**
Imipenem	25(25%)	_	75(75%)
Meropenem	25(25%)	_	75(75%)
Piperacillin+Tazobactam	37(37%)	_	63(63%)
Cefotaxim	93(93%)	_	7(7%)
Cefotriaxone	99(99%)	_	1(1%)
Cefoperazone	89(89%)	1(1%)	10(10%)
Ceftazidime	100(100%)	_	_
Cefepime	98(98%)	_	2(2%)
Aztreonam	58(58%)	_	42(42%)
Colistin	_	_	100(100%)
Polymyxin B	_	_	100(100%)
Gentamycin	49(49%)	_	51(51%)
Amikacin	47(47%)	4(4%)	49(49%)
Tobramycin	60(60%)	_	40(40%)
Ciprofloxacin	83(83%)	3(3%)	14(14%)
Amoxycillin clavulanic acid	99(99%)	_	1(1%)

Table 4: Distribution of MβL genes in 100 isolated *P. aeruginosa* strains by using PCR.

	Isolated **P. aeruginosa** *strains (No. 100)*	
MβL Genes	**Imipenem sensitive (No. 75)**	**Imipenem resistant (No. 25)**
IMP	--	--
VIM	--	13
SPM-1	--	--
SIM-1	--	--
GIM-1	--	--
VIM+ SPM-1	--	2

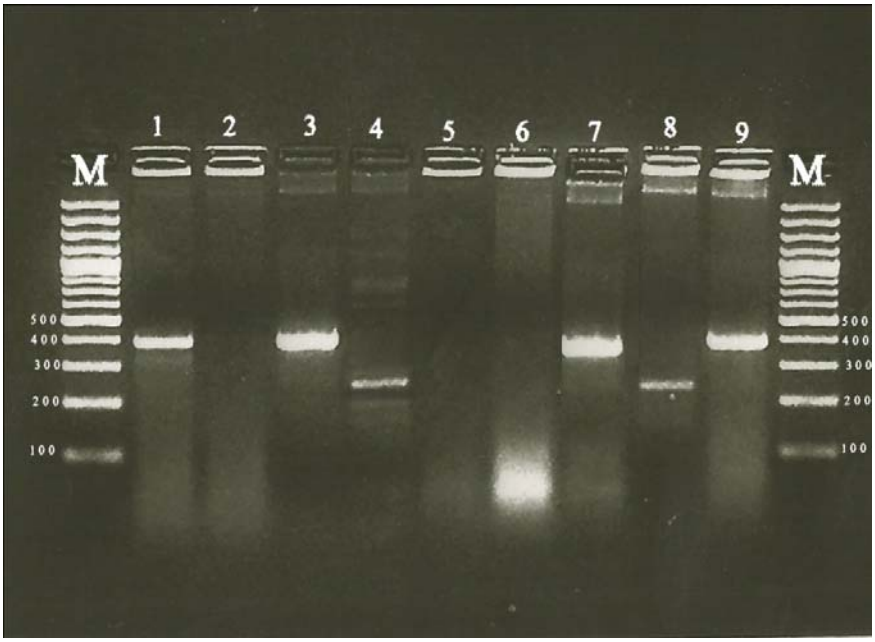

Fig 2: Gel electrophoresis of MβL genes in some strains of *P. aeruginosa*.

M is a ladder. Each two adjacent lanes represent two PCR reactions (M2-M1) for one strain of *P. aeruginosa*. Lanes (1,2) show one band with molecular weight 390 bp (*VIM* gene) (strain number 2).lanes (3,4) (strain number 1) and (7,8) (strain number 12) show a band with molecular weight 390 bp (*VIM* gene) and a band with molecular weight 271 bp (*SPM* gene). Lanes (5-6) are negative (strain number 20). Lane (9) shows a band with molecular weight 390 bp (*VIM* gene) (M2 reaction of strain number 30).

Table 5: Result of imipenem EDTA combined disc test in comparison with PCR For detection of MβLs in imipenem resistant *P.aeruginosa* strains.

PCR Combined disc test	***Positive***	***Negative***	***Total***	***Kappa value***
Positive	14	0	14	0.92
Negative	1	10	11	
Total	15	10	25	
Sensitivity = 93.3%		Specificity = 100%		
PPV = 100%		NPV = 90.9%		

Table 6: Antibiotic sensitivity pattern of isolated MβL producing *P. aeruginosa* strains.

Antibiotic	***MβL positive strains*** *(total 15)*		
	Resistant	**Intermediate**	**Sensitive**
Imipenem	15(100%)	_	_
Meropenem	15(100%)	_	_
Piperacillin+Tazobactam	11(73%)	_	4(27%)
Cefotaxim	15(100%)	_	_
Cefotriaxone	15(100%)	_	_
Cefoperazone	15(100%)	_	_
Ceftazidime	15(100%)	_	_
Cefepime	15(100%)	_	_
Aztreonam	13(87 %)	_	2(13%)
Colistin	_	_	15(100%)
Polymyxin B	_	_	15(100%)
Gentamycin	14(93 %)	_	1(7%)
Amikacin	12(80%)	_	3(20%)
Tobramycin	14(93 %)	_	1(7%)
Ciprofloxacin	15(100%)	_	_
Amoxycillin clavulanic acid	15(100%)	_	_

DISCUSSION

*P.aeruginosa*is one of the most important pathogens causing nosocomial infections; it is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms[15]. With the widespread use of extended-spectrum antibiotics, in a very short time span, *P. aeruginosa* has become resistant to a variety of antimicrobial agents, such as β-lactams, aminoglycosides, chloramphenicol, quinolones, tetracyclines and sulphonamides. Emerging resistance to expanded-spectrum cephalosporins and carbapenems among *P. aeruginosa* has been a major concern [16].

Over the last decade, MβL producing isolates have emerged particularly in *P.aeruginosa*. Poor therapeutic outcomes and increased mortality rates have been associated with infections of such organisms. Therefore, early detection of MβL producing organisms is crucial for optimal treatment of critically ill patients, to permit rapid initiation of strict infection control procedures and to prevent nosocomial spread [17].

So, this study was planned for evaluation of IMP-EDTA CDT as a phenotypic screening method for detection of MβL producing *P. aeruginosa*, detection of MβL genes in those isolates and formulation of antibiotic policy for treatment of infections by MβL producing *P.aeruginosa*.

In this study a total number of 100 *P.aeruginosa* isolates were recovered from 220 different specimens (Table 2) and the resistance rate of the isolated *P.aeruginosa* to different antibiotics was (25%) to imipenem and meropenem, (37%) to piperacillin+tazobactam, (93%) to cefotaxim, 99% to cefotriaxone, 89% to cefoperazone, 100% to ceftazidime, (98%) to cefepime, (58%) to aztreonam, (49%) to gentamycin, (47%) to amikacin, (83%) to ciprofloxacin, (99%) to amoxicillin-clavulanic acid and all isolated strains were sensitive to colistin and polymyxin B (Table 3).

The resistance rate of *P. aeruginosa* to different classes of cephalosporins in our study was very high. This may be due to the extensive use of them in our hospitals which made *P. aeruginosa* more resistant to them.

Another study conducted by *Gad et al.*[18] in Minia, Egypt, the resistance rates of isolated *P. aeruginosa* from clinical samples were 22% to meropenem, (68%) to cefotaxim, 49% to cefotriaxone, 36% to cefoperazone, (29%) to cefepime, (59%) to gentamycin, (8%) to amikacin, (29%) to ciprofloxacin, (91%) to amoxicillin-clavulanic acid.

Mansour et al.[19] in Zagazig university hospital in Egypt, found that the resistance rates of the isolated *P.aeruginosa* to different antibiotics were (49.2%) to imipenem and (50.8%) to meropenem, (62.7%) to ceftazidime, (76.3%) to cefepime, (96.6%) to aztreonam, (52.5%) to gentamycin, (54.2%) to amikacin, (64.4%) to ciprofloxacin and (67.8%) to tobramycin.

In a study by *Al-Agamy et al.* [20] in Saudi Arabia, the resistance rate of the isolated *P.aeruginosa* to different antibiotics was (38.5%) to imipenem and (38.2%) to meropenem, (50.3%) to piperacillin+tazobactam, (48.2%) to cefotaxim, (48.2)% to cefoperazone, (45%) to ceftazidime, (40.2%) to cefepime, (30.5%) to aztreonam, (40.3%) to gentamycin, (20.2%) to amikacin, (36.8%) to ciprofloxacin.

This difference could be attributed to the different rate of use of these antibiotics in different localities.

In the present study, out of 100 *P.aeruginosa* isolates, 25 isolates (25%) were imipenem resistant and 15 of them (60%) were carrying genes responsible for MβL production (15% of the total number of *P.aeruginosa*). Thirteen strains (13%) were carrying *VIM* gene while two strains (2%) were carrying both *VIM* and *SPM-1* genes together. *IMP, GIM-1, SIM-1* genes were not detected. None of the imipenem sensitive strains were carrying genes of MβL production (Table 4).

Our results coincide with another study in Egypt at Tanta University. *Essa and Afifi,*[21]**,** performed a study on 40 imipenem resistant and 20 imipenem sensitive strains of *P.aeruginosa.* They found that (40%) of the imipenem resistant isolates were positive for *VIM* gene and none of them had *IMP* gene and none of the imipenem sensitive isolates had any MβLs-gene.

In another study in Egypt, *Zafer et al.* [22] collected 122 *P. aeruginosa* isolates from hospitalized patients admitted to Kasr El-Aini Hospital and National Cancer Institute, Cairo University. The resistance rate to imipenem was (39.3%). Out of 122 *P. aeruginosa*, (27%) were MβL. The isolation rate of *VIM-2, NDM-,* and *IMP-1*-like genes were found in 58.3%, 4.2%, and 2.1%, respectively. *GIM-, SPM-, SIM-,* like genes were not detected in that study.

In Canada, *Pitout et al.* [17] reported that out of the 241 clinical strains of imipenem-nonsusceptible *P. aeruginosa* from the Calgary Health Region, 110/241 (46%) were MβL positive using phenotypic methods while 107/241 (45%) were PCR positive for MβL genes: 103/241 (43%) for *VIM* and 4/241 (2%) for *IMP*.

In Germany, *Valenza et al.* [23]**,** performed a survey in a German university hospital. They found that imipenem resistance was (13.9%) and the proportion of isolates producing MβL was (1.6%) with regard to all *P. aeruginosa* isolates investigated and (11.7%) with regard to the isolates with imipenem resistance. Molecular analysis revealed *VIM-1* and *VIM-2* genes.

In Iran, *Saderi et al.* [24] performed a study to detect isolation rate of MβL producing *P.aeruginosa* in burn patients. Among 100 strains of *P.aeruginosa*, 69/100 (69%) strains were imipenem resistant, 65/100 (65% of total strains) were MβL positive by phenotypic methods (94% of imipenem resistant) and 13/69 of imipenem resistant strains (18.8%) carried *VIM-2* gene. (*VIM-1*, *IMP-1, 2* were not detected).

Al-Agamy et al.[20], reported that imipenem resistance in Saudi Arabia is high (38.6%) and MβL is responsible for 20.57% of the resistance. All MβL producing *P. aeruginosa* were found to harbor *VIM*-2 gene while none of them carried *IMP, GIM-1, SIM-1* or *SPM-1* genes.

In a study by *Hammami et al.* [25] in Tunisia, 16 (67%) MβL-producer strains (out of 24 *P. aeruginosa*) were positive for *VIM-2* gene and one strain (4%) harboured the *IMP* gene.

In another study by *Polotto et al.* [26] in Brazil**,** they reported that (96.4%) of strains were imipenem resistant and the isolates harbouring MβL genes was (30.3%). (17.8%) presented the *SPM-1* gene and the *IMP-1* was detected in (12.5%).

Arunagiri et al. [16] in India reported that, out of 67 *P. aeruginosa* isolates, 62.7% (42/67) and 70.1% (47/67) were resistant to imipenem and meropenem respectively and 70.1% (47/67) were found to be MβL producers. Among this 47 MβL producing isolates, 41 (61.1%) strains carried *VIM* gene and 2 (3%) strains carried *IMP* gene.

In Algeria, *Touati et al.* [27]**,** performed a study on 17 imipenem resistant *P. aeruginosa* strains isolated from surgical intensive care unit at the University Hospital of Annaba. They found *VIM-2* gene in 14 out of 17 strains (82.3%).

This controversy in the incidence of strains carrying MβL-genes may be due to variations in the prevalence between different hospitals and geographic areas.

It was suggested that isolates with *VIM* gene have replaced those with *IMP* gene that were identified in the 1990s[28]. This agrees with the results of the present work that could not find *IMP* gene in any of the imipenem resistant isolates.

Since there are no standard guidelines for detection of MβLs, different studies have reported the use of different methods. PCR analysis is the gold standard method for the detection of MβL production, but it is not feasible in routine microbiology laboratory [29].

In the present study, we used IMP- EDTA CDT for screening for MβL production and comparing its result with the result of PCR and we found that the strength of agreement between them is very good *(Kappa value=0.92)(Table 5)* and the sensitivity & specificity of the IMP- EDTA CDT in relation to PCR was 93.3% and 100% respectively.

The results of our study are in agreement with the studies done by *Pandya et al.* [29] *and Arunagiri et al.* [16] who found that the sensitivity of IMP- EDTA CDT was (96.3%) & (94%) respectively, when using PCR as a gold standard.

In contrast, *Picao et al.* [30] reported lower sensitivity of IMP- EDTA CDT (80%) when compared with other methods of detection.

The resistance rates of the tested antimicrobial agents were higher for MβL-producing than non-MβL-producing *P. aeruginosa* isolates. In this study, the resistance rates in MβL-producing *P. aeruginosa*were (100%) to imipenem, meropenem, cefotaxim, cefotriaxone, cefoperazone, ceftazidime, cefepime, ciprofloxacin and amoxicillin clavulanic acid, (93%) to gentamycin and tobramycin, (87%) to aztreonam, (80%) to amikacin and (73%) to piperacillin+tazobactam and all isolated strains were sensitive to colistin and polymyxin B *(Table 6).*

Aztreonam is the only β-lactam that may remain fully active against MβL producers [9]. However high proportion (87%) of our strains was resistant to this β-lactam. This could be due to the existence of other mechanisms such as ESβL production, efflux pumps and cephalosporinase hyperproduction [9].

Zavascki et al.[31] in Brazil, agreed with our study. They reported that MβL producing *P.aeruginosa* were 100% resistant to imipenem, meropenem, ceftazidime and cefepime, 98.8% to ciprofloxacin, 88.4% to piperacillin/tazobactam, and 48.8% to aztreonam and all strains are sensitive to polymyxin B.

Al-Agamy et al. [20] reported that the resistance rate of the isolated MβL producing *P.aeruginosa* to different antibiotics was (100%) to imipenem, meropenem, cefotaxim, cefoperazone, ceftazidime, cefepime, piperacillin+tazobactam, (63.8%) to ciprofloxacin, (81.9%) to gentamycin, (68%) to amikacin, (16.6%) to polymyxin B and also they detected high resistant rate to aztreonam (63.8%).

In India *Ranjan, et al.* [32] reported that MβL producing *P.aeruginosa* were 100% resistant to meropenem, tobramycin and ciprofloxacin , 93% to imipenem, 29% to ceftazidime, 41% to cefepim, 41% to amikacin, 89% to gentamycin and 53% to aztreonam.

CONCLUSIONS

1) MβL producers is a serious problem as they are highly resistant to most antibiotics used while sensitive only to colistin and polymyxin B making treatment options very limited. Therefore, appropriate and regular screening system should be established specially for all imipenem resistant *P. aeruginosa* isolates for early detection of MβL producers and to prevent wider spread of such strains.
2) *VIM* gene is the most prevelant one in comparison with other genes of MβL production.
3) IMP-EDTA CDT is a sensitive method for detecting MβL production. It represents a valid alternative to the molecular investigation of MβL genes, making detection possible in routine diagnostic laboratories.

REFERENCES

1. Zhanel GG, DeCobry M, Adam H, Mulvey MR, Canadian Antimicrobial Resistance Alliance, Hoban DJ et al. Prevalence of antimicrobial resistant pathogens in Canadian hospitals: results of the Canadian Ward Surveillance Study (CANWARD 2008). Antimicrob. Agent Chemother., 2010; 54: 4684-4693.
2. Bonomo RA, Szabo D. Mechanisms of multidrug resistance in Acinetobacter species and P. aeruginosa. Clin. Infect. Dis., 2006; 43(2):S49-S56.
3. Pfeifer Y, Cullik A, Witte W. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. Int. J. Med. Microbiol., 2010; 300: 371-379.
4. Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M. Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. Antibiotics in Gram-Positive Bacteria. Federation of European Microbiological Societies (FEMS), 2008; 32(2):386-408.
5. Lee JH, Lee SH. Carbapenem resistance in gram-negative pathogens: Emerging Non-metallo-carbapenemases. Res. J. Microbiol., 2006; 1: 1-22.
6. Sacha P, Wieczorek P, Hauschild T, Zorawski M, Olszanska D, Tryniszewska E. MßL of P. aeruginosa: a novel mechanism resistance to ß-lactam antibiotics. Folia Histochem. Cytobiol., 2008; 46(2):137-42.
7. Marra AR, Pereira CA, Gales AC, Menezes LC, Cal RG, desouza JM. Blood stream infections with MBL- producing P. aeruginosa: Epidemiology, Microbiology and clinical outcomes. Antimicrob. Agents Chemother., 2006; 50: 388-390.
8. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect. Dis., 2011; 11(5): 355–62.
9. Walsh TR, Tolemenn MA, Poirel L, Nordman P. MβL; the quiet before the storm? Clin. Microbiol. Rev., 2005; 18(2): 306-325.
10. Qu TT, Zhang J, Wang J, Tao J, Yu YS et al. Evaluation of phenotypic tests for detection of MßL -producing P. aeruginosa strains in China. J. Clin. Microbiol., 2009; 47:1136-1142.
11. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty 2nd Informational Supplement. CLSI document M100S22. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute, 2012; 32 (3).
12. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem–EDTA disc method for differentiation of MßL-producing clinical isolates of Pseudomonas sp. and Acinetobacter sp. J. Clin. Microbiol., 2002; 40:3798-801.
13. Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired MβLs. J. Antimicrob. Chemother., 2007; 59:321-322.
14. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose Gel Electrophoresis for the Separation of DNA Fragments. J Vis Exp, 2012; 62: 3791–3923.

15. Hocquet D, Berthelot P, Roussel-Delvallez M, Favre R, Jeannot K, Bajolet O et al. P.aeruginosa may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. Antimicrob. Agents Chemother., 2007; 51: 3531–6.
16. Arunagiri K, Sekar B, Sangeetha G, John J. Detection and Characterization of MBL in P. aeruginosa by Phenotypic and Molecular Methods from Clinical Samples in a Tertiary Care Hospital. West Indian Med. J., 2012; 61(8): 778.
17. Pitout JD, Gregson DB, Poirel L, McClure J, Le P, Church DL. Detection of P. aeruginosa Producing MBL in a Large Centralized Laboratory. J. Clin. Microb., 2005; 43(7): 3129–3135.
18. Gad GF, El-Domany RA, Zaki S, Ashour HM. Characterization of P. aeruginosa isolated from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. J. Antimicrob. Chemother., 2007; 60: 1010–1017.
19. Mansour SA, Eldaly O, Jiman-Fatani A, Mohamed ML, Ibrahim EM. Epidemiological characterization of P. aeruginosa isolates of ICUs in Egypt and Saudi Arabia. Eastern Mediterranean Health Journal, 2013; 19(1): 71-80.
20. Al-Agamy MH, Shibl AM, Zaki SA, Tawfik AF. Antimicrobial resistance pattern and prevalence of MBL in P. aeruginosa from Saudi Arabia. African Journal of Microbiology Research, 2011; 30: 5528-5533.
21. Essa EA, Afifi IK. Multiplex PCR study of nosocomial P. aeruginosa for genes of MBL: could such enzymes bring us to end of antibiotics?. Egyptian Journal of Medical Microbiology, 2007; 16 (1):189-200.
22. Zafer MM, Al-Agamy MH, El-Mahallawy HA, Amin MA, Ashour MS. Antimicrobial Resistance Pattern and Their β-Lactamase Encoding Genes among P. aeruginosa Strains Isolated from Cancer Patients. BioMed Research International, 2014; Article ID 101635:1-8. http://dx.doi.org/10.1155/2014/101635
23. Valenza G, Joseph B, Elias J, Claus H, Oesterlein A, Engelhardt K et al. First Survey of MBL in Clinical Isolates of P. aeruginosa in a German University Hospital. Antimicrob. Agents Chemother., 2010; 54(8): 3493–3497.
24. Saderi H, Lotfalipour H, Owlia P, Salimi H. Detection of MβL- Producing P. aeruginosa Isolated From Burn Patients in Tehran, Iran. Lab. Med., 2010; 41(10): 609-12.
25. Hammami S, Boubaker IB, Ghozzi R, Saidani M, Amine S, Redjeb SB. Nosocomial outbreak of imipenem-resistant P. aeruginosa producing VIM-2 MBL in a kidney transplantation unit. Diagnostic Pathology, 2011; 6: 106
26. Polotto M, Casella T, Oliveira MG, Rúbio FG, Nogueira ML. Detection of P. aeruginosa harboring blaCTX-M-2, blaGES-1 and blaGES-5, blaIMP-1 and blaSPM-1 causing infections in Brazilian tertiary-care hospital. BMC Infectious Diseases, 2012; 12:176.
27. Touati M, Diene SM, Dekhil M, Djahoudi A, Racherache A, Jean-Marc RJ. Dissemination of a Class I Integron Carrying VIM-2 Carbapenemase in P. aeruginosa Clinical Isolates from a Hospital Intensive Care Unit in Annaba, Algeria. Antimicrob. Agents Chemother., 2013; 57(5) 2426–2427.
28. Gibb AP, Tribuddharat C, Moore RA, Louie TJ, Krulicki W, Livermore DM et al. Nosocomial outbreak of carbapenem-resistant P.aeruginosa with a new blaIMP allele, blaIMP-7. Antimicrob. Agents Chemother., 2002; 46:255–258.
29. Pandya NP, Prajapati SB, Mehta SJ, Kikani KM, Joshi PJ. Evaluation of various methods for detection of (MβL) production in gram negative bacilli. Int. J. Biol. Med. Res., 2011; 2(3): 775-777.
30. Picao RC, Andrade SS, Nicoletti AG, Campana EH, Moraes GC, Mendes RE et al. MßL Detection: Comparative Evaluation of Double Disk Synergy versus Combined Disk Tests for IMP-, GIM-, SIM-, SPM-, or VIM Producing Isolates. J. Clin. Microbiol., 2008; 46(6):2028-37.
31. Zavascki AP, Barth AL, Goncalves AL, Moro AL, Fernandes JF, Martins AF et al. The influence of MßL production on mortality in nosocomial P. aeruginosa infections. J. Antimicrob. Chemother., 2006; 58(2):387-392.
32. Ranjan S, Banashankari GS, Babu SPR. Comparison of Epidemiological and Antibiotic Susceptibility Pattern of MBL-Positive and MBL-Negative Strains of P.aeruginosa. Journal of Laboratory Physicians, 2014; 6(2): 109- 113.